REMARKS

Status of the claims

The response with amendments filed July 13, 2001 was received and entered. Claims 1-21 were presented for examination.

Election/Restrictions

Group I, claims 1-14, were elected in paper No. 6, filed July 13, 2001. Claims 15-21 were withdrawn from consideration as being drawn to a nonelected invention.

Drawings

The drawings are objected to, because Figure 1 misspells the first instance of the word "HORMONE". Although formal drawings need not be submitted at this time, correction is required.

In Figure 1, the misspelled word "HORMONRE" was changed to the word "HORMONE". A corrected copy of Figure 1 (as an informal drawing) is attached to this response on a separate page.

Claim rejections - 35 USC § 112, first paragraph

Claims 1-14 were rejected under 35 USC § 112, first paragraph, because the specification provides support for aminopeptidase isolated from *Aeromonas proteolytica*, but does not reasonably provide enablement for any *Aeromonas* aminopeptidase. Accordingly, the claims are not commensurate in scope with the enabling disclosure.

Claim 1 was amended by inserting the species name *proteolytica* to recite *Aeromonas proteolytica* aminopeptidase, limiting the scope of the claim.

Claim rejections - 35 USC § 102

Claims 1-8 and 10-14 were rejected under 35 USC 102(e) as being anticipated by Blumberg et al. (US 5,763,215 and EP 0 191 827 B1). Blumberg (US 5,763,215) is cited as disclosing a method of removing N-terminal amino acids from a variety of recombinantly produced polypeptides using an aminopeptidase isolated from *Aeromonas proteolytica*. Most of the disclosure (background, examples, and discussion) is directed to the problem of removing N-terminal methionine residues. Column 5, lines 53-57 also states that "These methods also apply, however, to the removal of N-terminal methionine from the terminus of the alanine form of bGH, which contains an alanine on the N-

terminus of its natural form although in this case the alanine residue may also be removed." This enzyme is reported to be active at pH values from 7 to 10, with pH 9.5 being most preferred, and stable to 65°C, with 37°C being most preferred. The enzyme may be bound to a solid support such as agarose or other polymeric resin or removed from the product by an affinity resin, if used in solution. Tris and borate buffers were used in the examples.

The focus of Blumberg is to show that *Aeromonas* aminopeptidase can be used to remove amino-terminal methionine residues from the ends of recombinant eukaryotic proteins. These amino terminal methionine residues may be undesireable, provoking an immune response if a recombinant protein is administered as a therapeutic, compared to a similar molecule, recombinant or not, that lacks such a residue.

Blumberg notes that "preliminary results indicate that not all proteins are susceptible to attack by *Aeromonas proteolytica* aminopeptidase." At the same time, Blumberg is inconsistent in its statements, and its experimental results as to whether *Aeromonas* aminopeptidase (AAP) can be used to remove other amino acids, particularly alanine from the amino terminus of proteins.

Example X of Blumberg illustrates that removal of methionine from Met-PGH is inefficient (4.63 nmoles removed, compared to a theoretical maximum of 7.31 nmoles, or ~60%) and that the removal of alanine from human superoxide dismutase (hSOD) is very inefficient (0.15 nmoles removed, compared to a maximum of 10.17 nmoles expected) after a 22 hour incubation period.

The source of the recombinant hSOD cited in example X of Blumberg is not explicitly stated, other than to say that the recombinant protein has the authentic N-terminal sequence of the mature protein and that the N-terminal Ala is not N-acetylated. One of many entries found in GenBank is shown below:

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>sp|P00441|SODC_HUMAN SUPEROXIDE DISMUTASE [CU-ZN] (EC 1.15.1.1) - Homo sapiens
(Human).
ATKAVCVLKGDGPVQGIINFEQKESNGPVKVWGSIKGLTEGLHGFHVHEFGDNTAGCTSA
GPHFNPLSRKHGGPKDEERHVGDLGNVTADKDGVADVSIEDSVISLSGDHCIIGRTLVVH
EKADDLGKGGNEESTKTGNAGSRLACGVIGIAQ
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The inefficient removal of Met from Met-PGH and Ala from hSOD illustrated in Table VI of example X are not adequately discussed, and Blumberg offers no reliable ways to predict when methionine, alanine, or any other residue will be efficiently removed from the amino terminus of proteins by AAP.

These direct results *teach away*, in fact, from the mere statement in column 5, lines 53-57 of Blumberg, which reads "These methods also apply, however, to removal of N-terminal methionine

from the terminus of the alanine form of bGH, which contains an alanine on the N-terminus of its natural form although in this case the alanine residue may also be removed." Blumberg, in fact, does not disclose *any data* to support the notion that alanine can be removed from bGH, or less any other recombinant protein. The phrase "may also be removed" is speculative in this context, and inconsistent with other experimental data (cited above) and discussion throughout the Blumberg reference.

Blumberg also comments on the decreased efficiency of methionine removal for certain substrates at column 18, lines 38-59. Met-Leu-hGH and Met-bGH, for example, showed about 65% of Met released/mole substrate. This efficiency was lower, 50-60%, for Met-pGH. Blumberg attributes the partial or incomplete stoichiometry to impure starting materials, incomplete removal of the N-formyl methionine group by the *E. coli* host deformylating enzymes, dimer formation of some hormones limiting access of the enzyme to one of the monomers in a dimer, and/or partial removal of the N-terminal methionine by the host cell before purification of the substrate proteins. At column 16, lines 65-67, Blumberg also states that further experiments would be required to prove any of these or other possibilities. These comments, taken together, suggest that other statements about the ability of AAP to remove one or more amino acids, including alanine, from the amino terminus of a protein are merely speculative.

The experimental results disclosed in the present application, however, clearly show that AAP can be used to remove N-terminal alanine residues from several exemplary proteins, under the conditions described therein.

Claims 1-2 and 14 were also rejected under 35 USC 102(e) as being anticipated by Pedersen et al. (US 5,783,413). Pederson is reported to disclose a method of removing amino terminal residues from peptides and polypeptides by means of *Aeromonas proteolytica* aminopeptidase. This enzyme is reported to have broad specificity, but is blocked by Pro and pyroGlu. The enzyme is also apparently blocked when Glu, Pro, Gln, or Asp residues are on the N-terminus of the protein substrate (See claim 7 of US 5,783,413).

Pedersen is directed to a multistep enzymatic process for producing a desired protein from an amino terminally extended protein of the formula A-Glutamine-Protein, where A is an amino acid attached to the glutamine residue, and the extended protein is reacted with an aminopeptidase, glutamine cyclotransferase, and pyroglutamine aminopeptidase. Pederson does not discuss removal of alanyl residues from the amino terminal residue of a protein, where A is alanine and the amino acid preceding the desired protein is not glutamine. Pederson also does not provide any specific

guidance as to the conditions needed to remove alanyl residues from the amino terminal residue of a protein by *Aeromonas proteolytica* aminopeptidase.

Claim rejections - 35 USC § 103

Claims 1-14 were rejected under 35 USC 103(a) as being unpatentable over Blumberg et al (US 5,763,215) and Pedersen et al. (US 5,783,413) in view of Harper et al. (US 4,900,673) and Obata et al. (JP 07,289,256) or Obata et al. (1997).

The reported teachings of Blumberg et al. and Pedersen et al. were cited above. Neither reference cites use of phosphate or CHES buffer.

Harper et al. is cited as disclosing AAP to remove Met from a precursor of angiogenin at pH 7.2 in phosphate buffer (Example 9).

Both Obata et al. references are cited as disclosing the isolation and characterization of an aminopeptidase from the marine bacterium *Aeromonas salmonicidia* having a high specificity for L-alanine (aminopeptidase K), having a pH optimum of about 6.5, but stable from 7-10, and a temperature optimum of 45°C.

Obata et al. (1997) briefly describes the various types of aminopeptidases (α-aminoacyl-peptide hydrolases) that can selectively hydrolyze specific amino acid residues, including proline aminopeptidase, glutamate aminopeptidase, aspartate aminopeptidase, lysine aminopeptidase, glycine aminopeptidase, and leucine aminopeptidase, etc. Obata also goes onto say that no aminopeptidases that specifically release N-terminal alanine or arginine residues have been reported from a peptide-degrading bacterium.

The unexpected discovery that *Aeromonas proteolytica* aminopeptidase, has the ability to remove alanine residues from the amino terminus of peptides and polypeptides, however, facilitates the ability of researchers to use this enzyme to remove undesired amino acid residues from recombinantly produced precursor polypeptides. *Aeromonas proteolytica* aminopeptidase can be obtained from commercial sources (e.g., Sigma Chemical Company).

The aminopeptidase K, isolated from *Aeromonas salmonicidia*, is unlikely to be produced on a commercial scale. While Obata et al. (1997) reported the optimum pH and pH stability, optimal temperature and heat stability, K_m and V values, and the effect of various protease inhibitors on the activity of this enzyme, substrate specificities were only tested with a variety of synthetic peptides

(e.g., L-Ala-βNA, Gly-βNA, and L-Pro-βNA). The hydrolytic activity of the aminopeptidase K was not reported on a variety of polypeptides containing an amino-terminal alanine residue.

The general conditions, then, that may be optimal for hydrolytic activity on a variety of synthetic substrates may not be optimal for processing of residues positioned at the amino terminus of a much longer polypeptide. Optimal reaction conditions reported for the cleavage of synthetic peptide substrates by *Aeromonas salmonicidia* aminopeptidase K, may not be extrapolatable to conditions favoring alanine removal by *Aeromonas proteolytica* aminopeptidase on a variety of polypeptide substrates.

To overcome these rejections, Claim 1 was amended to incorporate the pH range limitation of claim 4. Claim 4 was canceled and Dependent Claim 5 amended to recite Claim 1 instead of Claim 4

Claims 1, 2, and 3 were also amended to replace the limiting phrase "recombinant protein" with the word "protein". The method of removing alanine residues at the amino terminus of a polypeptide is not solely limited to polypeptides produced by recombinant means.

The parenthetical expression in claim 14, "... wherein the aminopeptidase is not immobilized (carried out in free solution)" was also deleted.

In view of the above, it is submitted that the claims are in condition for allowance. Reconsideration of the rejections and objections is requested.

Claim 4 was canceled. Claims 1, 2, 3, 5, and 14 were amended. No new Claims were added. Claims 1-3 and 5-14 are pending in the application. A complete set of the pending claims is appended to this response. Allowance of the pending claims an early date is solicited.

Respectfully submitted,

Verne A. Luckow

Verne A. Luckow
Attorney for Applicants
Registration No. 45,950
(314) 694-6993 (Creve Coeur, MO)

Global Patent Department
Pharmacia Corporation
800 N. Lindbergh Blvd., O4E
St. Louis, Missouri 63167

Entire set of claims with annotations

Cancel Claim 4.

Amend the claims 1, 2, 3, 5, and 14 as follows:

1. (Once amended) A method of removing an N-terminal alanyl group from a [recombinant] protein which comprises contacting said [recombinant] protein with *Aeromonas* *proteolytica* aminopeptidase such that said N-terminal alanyl group is removed and recovering the resulting [recombinant] protein, wherein said contacting is carried out at a pH from about pH 7 to about pH 11.

2. (Once amended) A method of claim 1 wherein said [recombinant] protein is of eukaryotic origin.

3. (Once amended) A method of claim 2, wherein said [recombinant] protein is selected from the group consisting of human growth hormone (HGH), bovine somatotropin (bST), porcine somatotropin (pST), and human tissue factor pathway inhibitor (TFPI).

4. ~~(Canceled) A method of claim 1, wherein said contacting is carried out at a pH from about pH 7 to about pH 11.~~

5. (Once amended) A method of claim 1[4], wherein said contacting is carried out at a pH from about pH 8 to about pH 10.

6. A method of claim 5, wherein said contacting is carried out at a pH of about pH 8.0 to about pH 9.5.

7. A method of claim 1, wherein said contacting is carried out in the presence of a buffer selected from the group consisting of borate, CHES, sodium bicarbonate, sodium phosphate, and Tris-HCl.

8. A method of claim 7, wherein said buffer is borate.

9. A method of claim 7, wherein said buffer is sodium phosphate.

10. A method of claim 7, wherein said buffer is Tris-HCl.

11. A method of claim 1, wherein said aminopeptidase is immobilized.

12. A method of claim 11, wherein said aminopeptidase is immobilized on a chromatography resin, chromatography surface, or chromatography gel.

13. A method of claim 12 wherein said recombinant protein is passed through a column containing said aminopeptidase immobilized on a chromatography resin.

14. (Once amended) A method of claim 1, wherein said aminopeptidase is not immobilized [(carried out in free solution)].

Claims 15-21 were withdrawn from consideration

15. A recombinant protein lacking an N-terminal alanine prepared by the method of claim 1.

16. A method of removing amino-terminal amino acids from a precursor polypeptide of the formula X-Y-Pro-Z with *Aeromonas* aminopeptidase to yield a polypeptide of the formula Y-Pro-Z, wherein X is one or more amino acids except proline, Y is any amino acid except proline, and Z is one or more amino acids.

17. The method of claim 16 wherein X is alanine.

18. The method of claim 16 wherein Y is selected from the group consisting of phenylalanine, methionine, threonine, and aspartic acid.

19. The method of claim 18, wherein Y is phenylalanine.

20. The method of claim 16, wherein X is alanine and Y is phenylalanine.

21. The method of claim 19 or claim 20, wherein the precursor polypeptide is Ala-hGH.